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Morphogenesis of the reproductive shoots of *Welwitschia mirabilis* and *Ephedra distachya* (Gnetales), and its evolutionary implications

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Abstract

For decades, Gnetales appeared to be closely related to angiosperms, the two groups together forming the anthophyte clade. At present, molecular studies negate such a relationship and give strong support for a systematic position of Gnetales within or near conifers. However, previous interpretations of the male sporangiophores of Gnetales as pinnate with terminal synangia conflict with a close relationship between Gnetales and conifers. Therefore, we investigated the morphogenesis of the male reproductive structures of *Welwitschia mirabilis* and *Ephedra distachya* by SEM and light microscopy. The occurrence of reduced apices to both halves of the antherophores of *W. mirabilis* gives strong support for the assumption that the male 'flowers' of *W. mirabilis* represent reduced compound cones. We assume that each half of the antherophore represents a lateral male cone that has lost its subtending bract. Although both halves of the antherophores of *Ephedra distachya* lack apical meristems, the histological pattern of the developing antherophores supports interpreting them as reduced lateral male cones as well. Therefore, the male sporangiophores of Gnetales represent simple organs with terminal synangia. Although extant conifers do not exhibit terminal synangia, similar sporangiophores are reported for some Cordaitales, the hypothetical sister group of conifers. Moreover, several Paleozoic conifers exhibit male cones with terminal sporangia or synangia. Therefore, we propose that conifers, Cordaitales and Gnetales originated from a common ancestor that displayed simple sporangiophores with a terminal cluster of sporangia.

Keywords: Gymnosperms; Gnetales; *Welwitschia*; *Ephedra*; Morphology; Male; Cones; Phylogeny

Introduction

Despite numerous investigations in the past 100 years, the systematic position of the Gnetales is still poorly understood. In most morphological analyses a close relationship between Gnetales and angiosperms was assumed, even though several fundamental differences remained unexplained. In the last 10 years, morphological analyses, including the fossil record, have given further support to an anthophyte clade related to the extinct Bennettitales, Pentoxylales, and Caytoniales (reviewed in Doyle 1998).

Early molecular analyses, based, e.g. on *rbc*L sequences, preliminarily supported the anthophyte group, but with low statistical support (Chase et al. 1993). In contrast, most molecular analyses of the past 5 years rejected a close relationship between Gnetales and angiosperms (Winter et al. 1999; Bowe et al. 2000; Chaw et al. 2000; Frohlich and Parker 2000; Schmidt and Schneider-Poetsch 2002). However, the results of these studies are controversial: Gnetales are sometimes placed at the base of all other gymnosperms (Rydin et al. 2002; Schmidt and Schneider-Poetsch 2002), or they are linked

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with conifers (Winter et al. 1999; Bowe et al. 2000), whereas some analyses support a close relationship to Pinaceae (Chaw et al. 2000; Gugerli et al. 2001).

It is worth noting that Eames (1952) had proposed a diphyletic situation for Gnetales, with *Ephedra* related to conifers, the remaining Gnetales (*Welwitschia* and *Gnetum*) as sister to angiosperms. Today, the monophyly of Gnetales is established, with *Ephedra* as the sister to *Gnetum* + *Welwitschia* (Doyle 1996; Price 1996). Most of the putative synapomorphic characters of the anthophyte clade occur in the supposedly derived genera *Gnetum* and *Welwitschia*, and the considerable heterogeneity of Gnetales is the main obstacle for a convincing interpretation of their morphology. Therefore, reanalysis and re-evaluation of the morphology of Gnetales seems to be necessary (Donoghue and Doyle 2000).

The crucial question in the discussion of the phylogeny of Gnetales is the interpretation of the male 'flower', or antherophore, in *Welwitschia* and *Ephedra*. The interpretation of the antherophore of *Ephedra* as a compound of two fused, pinnate sporangiophores is largely accepted (Hufford 1996). In the past, the antherophore of *Welwitschia* was interpreted in different ways (reviewed by Endress 1996; Hufford 1996). One possibility is to regard the antherophore like that of *Ephedra*, i.e. as two fused, pinnate sporangiophores, each with three stalked synangia (McNab 1873; Martens 1975). Another interpretation is to consider the antherophore as a kind of androecium, consisting either of one whorl of six stamens (Hooker 1863), or of two whorls with two outer and four inner stamens (Church 1914; Strasburger 1872). A third interpretation is to regard the antherophore as composed of two fused, axillary, simple male cones (Lignier and Tison 1911). Today, the first interpretation is mostly favoured, because the antherophore develops from two distinct primordia that continue the decussate phyllotaxis of the flower. Following this interpretation, the antherophores in *Ephedra* and *Welwitschia* are homologous structures that represent two pinnate sporangiophores (Hufford 1996).

This interpretation makes a close relationship between Gnetales and conifers difficult to understand, as the male sporangiophores of the latter are simple and the sporangia not located in a terminal position. The stamens of most angiosperms are also simple, but they share with those of the Gnetales a terminal position of the synangia, and in some cases (e.g. *Typha latifolia*) the filaments are fused at the base, forming a 'columnar structure' (Hufford 1996). The sporangiophores of the cycads could also be regarded as pinnate structures, but the position of the synangia is completely different (Mundry and Stützel 2003). Thus, the relationship between the sporangiophore of the Gnetales and other living seed plants is equivocal.

The fossil record of Gnetales, especially of the male reproductive structures, is also ambiguous. The oldest putatively gnetalean macrofossils are the early Mesozoic male cones *Masculostrobus clathratus* and *Piroconites kuespertii* (Ash 1970; van Konijnenburg-van Cittert 1992). In both species, the typical striate, ephedroid pollen has been found in situ, and both cones are associated with leaves that display a decussate arrangement. Moreover, the male sporangiophores of *P. kuespertii* bear trisporangiate synangia on the proposed abaxial side (Ash 1970; van Konijnenburg-van Cittert 1992). Despite these similarities to extant Gnetales, the macromorphology of both species is quite different, and some authors assume that the occurrence of ephedroid pollen has been overemphasized for the confirmation of a gnetalean relationship (Hughes 1994). Further, early Mesozoic male reproductive structures assigned to Gnetales are *Dinophyton* and *Sanmiguelia*, but their relationship to Gnetales seems equivocal (Crane 1996). From the Cretaceous, some macrofossils have been found (e.g. *Drewria*, see Crane and Upchurch 1987), but morphological details of the male cones are not preserved or have not yet been investigated.

To better understand the morphology of Gnetales, we investigated the early morphogenesis of the male 'flowers' in *Welwitschia mirabilis* and *Ephedra distachya* by SEM and light microscopy. Based on our results, the homology of sporangiophores among extant and fossil gymnosperms is discussed in order to gain new insights into their relationship.

Material and methods

W. mirabilis Hook. f. and *E. distachya* L. were studied throughout two years in the Botanical Garden of the University of Bochum, Germany. The male cones of *W. mirabilis* were collected at irregular intervals during late spring and summer, those of *E. distachya* were harvested from autumn to spring. Immediately after the cones were collected, they were dissected under a stereomicroscope and fixed in FAA (formalin, acetic acid, ethanol 70%; 5:5:90). The fixed material was dehydrated in 70% ethanol and immersed in formaldehyde-dimethylacetal for at least 24 h (Gerstberger and Leins 1978). After critical-point drying, the specimens were examined with a DSM 950 SEM (Zeiss), and the results documented using Digital Image Processing Software 2.2 (DIPS-Leipzig). For the male cones of *W. mirabilis*, the duration of intermedium exchange was extended (at least 48 h) and the gas-out was carried out very slowly (at least 1 h) to avoid collapse of the tissues. Transverse and longitudinal serial sections of male flowers of different developmental stages were made using a classical paraffin-technique and Astrablue–safranin

staining (Gerlach 1984). Microtome sections of 10 µm thickness were examined using a Zeiss Axioplan light microscope, and the results documented using the software AnalySIS (Soft-Imaging-Systems). The male flowers were cleared with 12–14% sodium hypochlorite and examined using a Wild M400 stereoscope with transmitted light. The results were documented using a Nikon Coolpix 990 digital camera.

Remarks on terminology

The published descriptions and discussions of gnetalean morphology are dominated by the supposed relationship to the angiosperms. The term antherophore (a structure bearing anthers) implies homology, or at least a close relationship, to the androecium of the angiosperms. We have adopted this term in order to avoid the introduction of new terms, but this should not be seen as an assumption of homology. In the same way, we use the term flower for a simple cone, without implying its homology to the angiosperm flower, although we avoid the term perianth for the sterile bracts of the flower. On the basis of our previous studies (Mundry and Mundry 2001), and to avoid loss of comprehensibility, we prefer the neutral term sporangiophore instead of sporophyll for the sporangia-bearing structures, because the homology of these structures is ambiguous, even though they may take the position of leaves. In this regard we apply the term 'phylloid tip' to the distal, leaf-like parts of sporangiophores.

Results

Macromorphology of the male 'flower' of *Welwitschia mirabilis*

The male specimen of *W. mirabilis* studied is about 25 years old. The stem is about 20 cm in diameter. Each year, about 4–6 fertile axes arise at the bases of the two persistent leaves (Fig. 1A). The fertile axes bear several male cones, each comprising up to 80–100 male 'flowers' (Fig. 1B). The male 'flowers' are about 5 mm long and 3 mm wide, and are borne in the axils of bracts of the cone axis (Fig. 1C). Each 'flower' consists of two pairs of decussate bracts, a tubular antherophore, and a terminal, sterile ovule. The antherophore usually bears 6 stalked synangia that are tri-sporangiate, exceptionally tetra-sporangiate. The inner pair of the bracts encloses the antherophore and the sterile ovule until anthesis. The blooming sequence is acropetal. Anthesis of the cones begins in early summer and may last for 3 months, each single flower blooming for a few days.

Morphogenesis of the male 'flower' of *Welwitschia mirabilis*

The male cones begin to develop in spring. The cone apex is about 200 µm high and has a diameter of about 200 µm at the base (Fig. 2A). The longitudinal sections of the cone dome show periclinal divisions of the surface layer. Thus, a subapical group of initials is formed (sa, Fig. 3B). From the flanks of this subapical group a peripheral tissue zone and from the centre of this group a central tissue zone originate. The cells of the peripheral zone are small and densely cytoplasmic, whereas the cells of the central tissue zone begin to vacuolate and later form the pith of the cone axis.

At the base of the cone dome the bracts are initiated in decussate sequence. The primordia of each decussate pair are laterally united and form one ring-like primordium (Fig. 2A). The microtome sections show that initiation of the primordia begins with periclinal divisions of the dermal and hypodermal layers (Fig. 3A and B). Thereafter, the dermal layer undergoes only anticlinal divisions. It becomes more and more vacuolated and shows more and more typical epidermal features.

In the axils of the bracts, the male 'flowers' are initiated beginning with an elliptical apex of about 100 µm length (Fig. 2A). Afterwards two bracts (tb) are formed at the base of the male 'flower' in transversal position (Fig. 2B). Formation of the next organ begins with the initiation of two lateral and two median, small primordia (arrows, Fig. 2B). During further development, these four primordia are fused to form one ring-like primordium (Fig. 2C). At this developmental stage, the reproductive apex of the male 'flower' is about 100 µm long and high. As seen in longitudinal sections (Fig. 3C), the apex of the male 'flower' shows a histological pattern similar to the apex of the cone dome, although the apex of the male 'flower' is not as massive. Likewise, there are several periclinal divisions in the dermal layer, which form a subapical group of initials. The peripheral and central tissues again originate from this group.

During further development, the median parts of the ring-like primordium continue to grow, forming the two laterally fused median bracts (Fig. 2C and D). Subsequently, two primordia of the antherophore are initiated at right angles to the inner bracts (Fig. 2D). Each primordium has a triangular shape with a diameter of about 100 µm, forming one-half of the developing antherophore. Longitudinal sections of the primordia show several periclinal divisions in the dermal layer and the formation of a small group of subapical initials. A weak zonation into peripheral and central tissues is present (Fig. 3D).

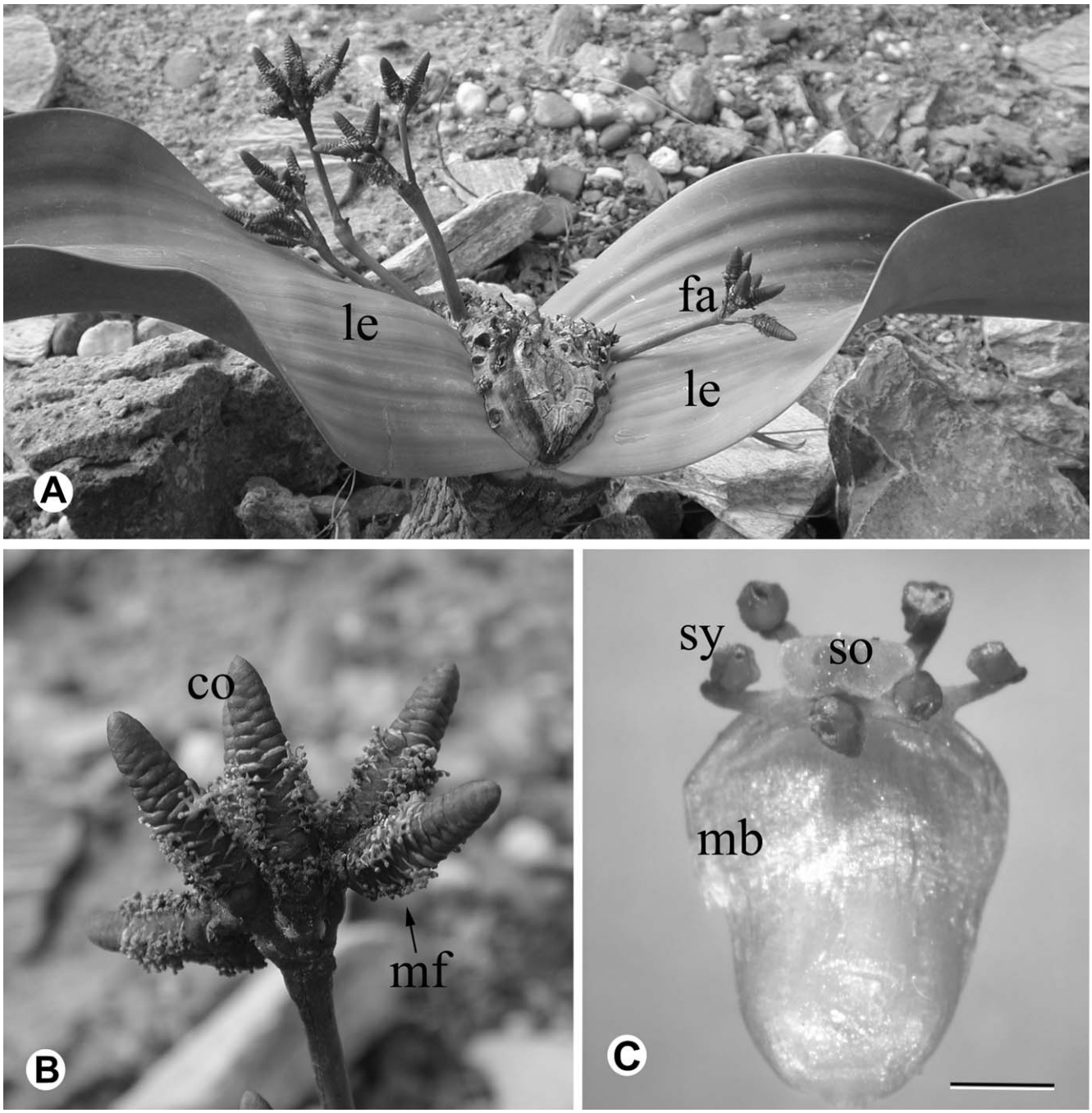


Fig. 1. *Welwitschia mirabilis*: (A) Male plant with the two persistent leaves and several fertile shoots. (B) Fertile shoot with several male cones. (C) Male 'flower' with median bracts surrounding the antherophore and the sterile ovule. Scale bar: 1 mm. co = cone; fa = fertile axis; le = leaf; mb = median bract; mf = male 'flower'; so = sterile ovule; sy = synangium.

At the base of both halves of the antherophore, the stalked synangia are initiated (Fig. 4A). Each half forms three synangia in a whorled, trimerous arrangement. In the centre of each half of the antherophore an apical meristem initially remains. At this developmental stage, the reproductive apex of the male 'flower' begins to differentiate the sterile ovule forming the integument (Fig. 4B). During further development, the primordia of the synangia enlarge, whereas the apical meristems of both halves of the antherophore stop growing and then abort (Fig. 4B: am, 4C: arrow). Longitudinal sections show that the apical meristem arrests its development when the synangia are initiated, as indicated by the less dense cytoplasm (Fig. 3F).

At first, the primordia of the synangia show some periclinal divisions in the dermal layer, but later on only

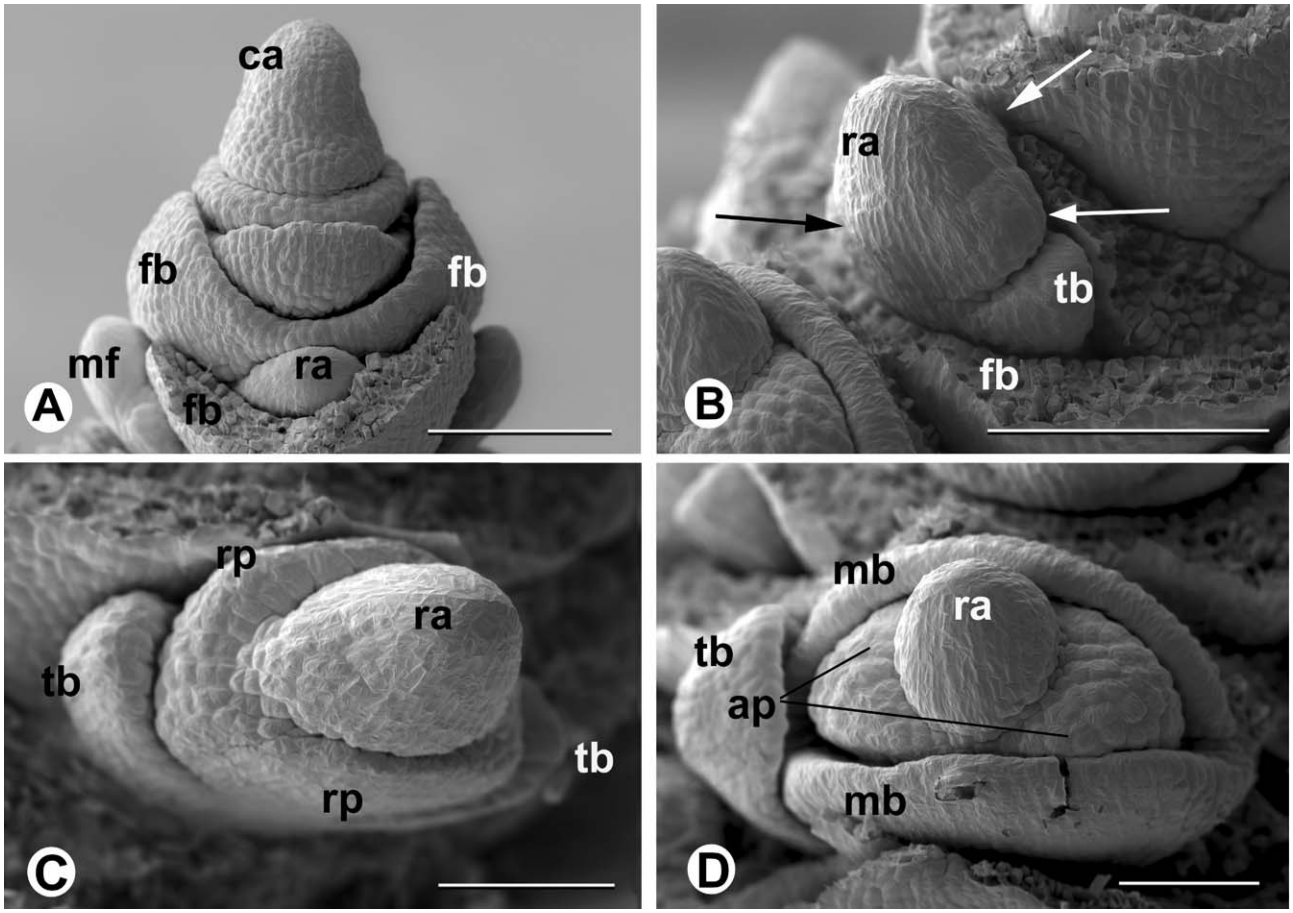


Fig. 2. *Welwitschia mirabilis*, morphogenesis of male cones; SEM micrographs. (A) Cone apex in spring, with several fertile bracts. (B) Reproductive apex of male 'flower' arising in axil of a fertile bract. (C) Male 'flower' with two transversal and two median bracts. (D) Male 'flower' with developing sporangiophores consisting of two primordia in transversal position. Scale bars: A,B 200 μm, C,D 100 μm. ap = antherophore; ca = cone apex; fb = fertile bract; mb = median bract; mf = male 'flower'; ra = reproductive apex; rp = ring-primordium; tb = transversal bract.

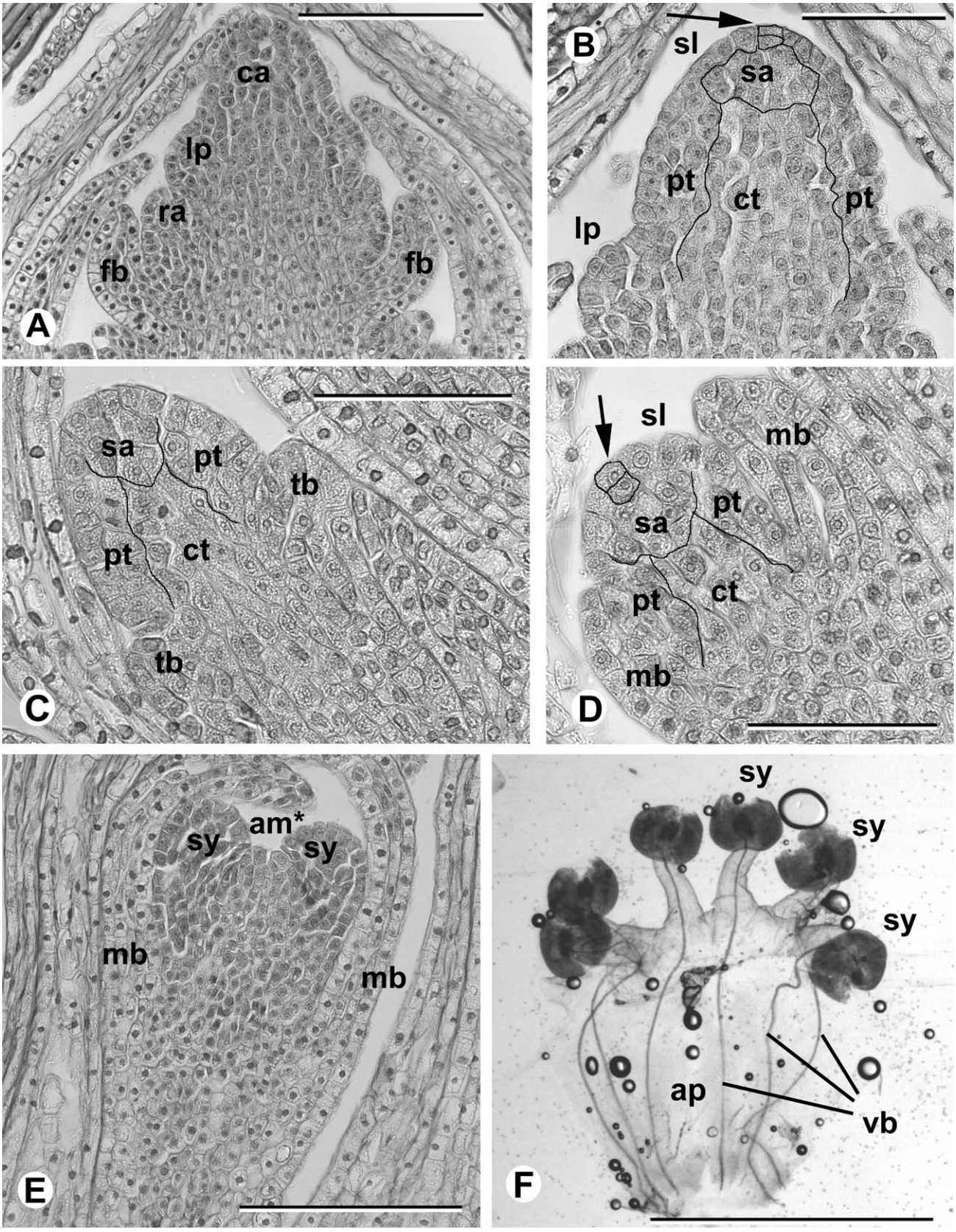
anticlinal ones. Subsequently, the sporogenous tissue originates from hypodermal layers (Fig. 3E). The intercalary growth zone at the base of both halves of the antherophores enlarges so that the halves become united and acquire their final tubular shape. Each synangium is supplied by a single vascular bundle (Fig. 3F). Usually three sporangia per synangium are formed, with three radial dehiscence lines (Fig. 4D). In some cases, four sporangia per synangium are formed, with the dehiscence lines arranged crosswise.

Macromorphology of the male cones of *Ephedra distachya*

The investigated male specimen of *E. distachya* is ca. 25 years old, 0.5 m tall and has a circumference of about 4 m. The male cones arise in the axils of bract-like leaves of the long shoots. They are either simple, in terminal position on the short shoot, or compound with one terminal and usually two lateral, simple cones arising in the axils of the basal bud scales. The male cone consists of 2–3 pairs of sterile bracts and 2–3 fertile bracts bearing the antherophores (Fig. 5A). The antherophores are sheathed by two united bracts in a median position. Each antherophore consists of about eight disporangiate synangia in terminal position (Fig. 5B). The dehiscence lines of the fused sporangia face one another; pollen is released in spring.

Morphogenesis of the simple cone of *Ephedra distachya*

Morphogenesis of the compound male cone begins in autumn with the formation of an apex 150 μm in diameter. The apex forms 2–3 pairs of sterile bud scales. Longitudinal sections of the cone dome show a partition into a dermal layer displaying only anticlinal cell divisions and a group of subapical cells displaying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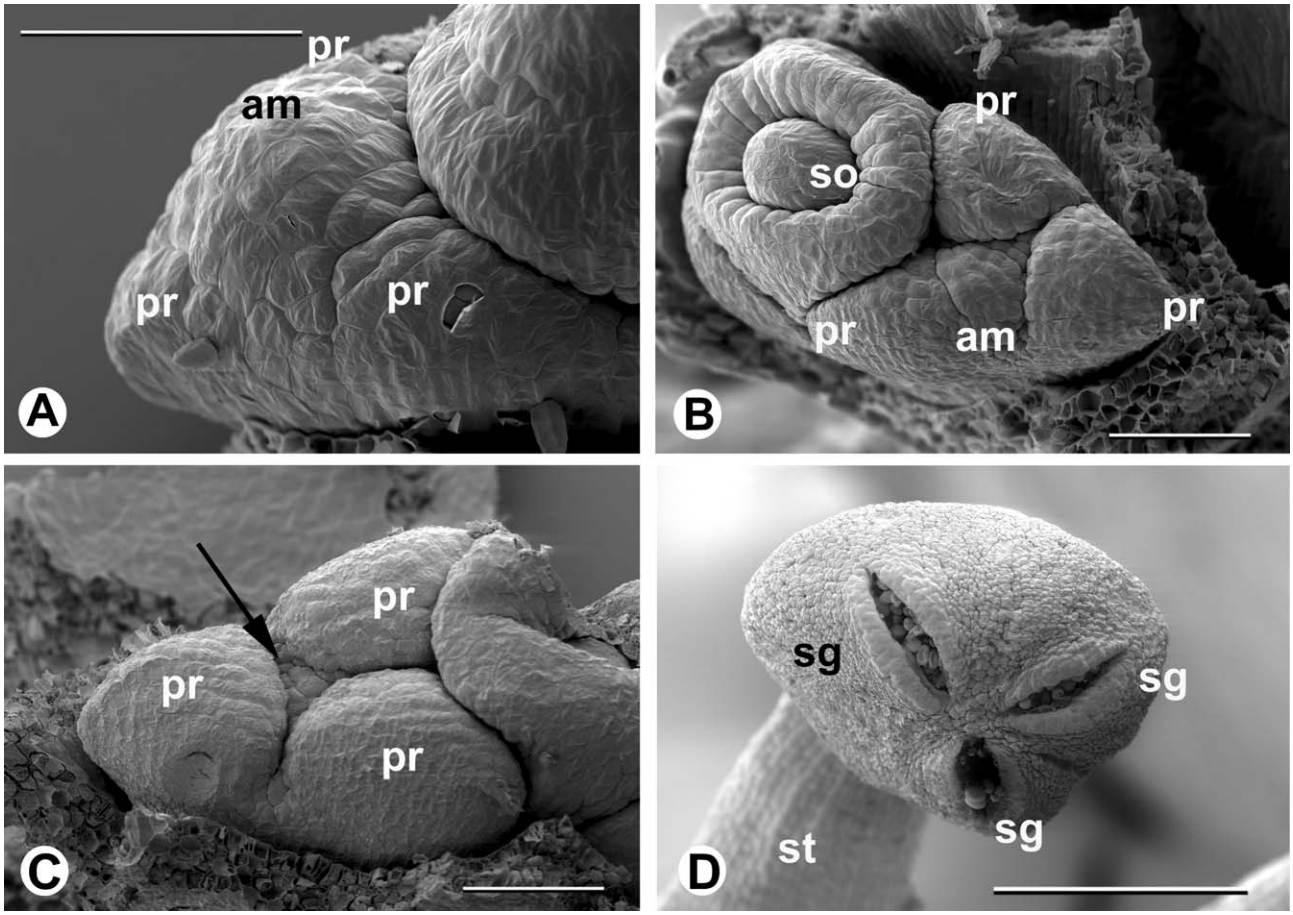


Fig. 4. *Welwitschia mirabilis*, morphogenesis of male 'flower'; SEM micrographs. (A) One primordium of the antherophore in detail; at the base of the apical meristem, the primordia of stalked synangia are initiated. (B) At the time of initiation of the ovule, the apical meristem stops growing and the primordia overtop it. (C) During further development, the primordia are enlarged whereas the apical meristem aborts (arrow). (D) Each synangium forms three sporangia, their dehiscence slits point to the centre of the synangium. Scale bars: A–C 100 µm, D 200 µm. am = apical meristem; pr = primordium; sg = sporangium; so = sterile ovule; st = stalk.

periclinal divisions. The peripheral tissue zone with dense cytoplasm and the central tissue zone with large vacuoles originate from this subapical group (Fig. 7A).

After initiation of the sterile bud scales, 2–3 pairs of fertile bracts are formed in a decussate arrangement. The reproductive apices in the axils of these bracts (Fig. 6A) are triangular in shape with an edge length of about 50 µm. The first two organs formed by this apex are in opposite median position (Fig. 6B: mb). The adaxial one precedes the abaxial in development, and both together later form the sheath of the antherophore. At right angles to these, two large primordia are then formed (Fig. 6B: pr), leaving only a minor rudiment of the former apex (Fig. 6B: ra).

These primordia are circular, about 150 µm in diameter. Their growth originates from a series of

Fig. 3. *Welwitschia mirabilis,* morphogenesis of male cones; LM micrographs. (A) Longitudinal section of cone apex with reproductive apices arising in axils of fertile bracts. (B) Cone apex in detail, displaying periclinal divisions of the surface layer, the subapical group, the peripheral and the central tissue zone. (C) Longitudinal section of reproductive apex of male 'flower' with two transversal bracts; the reproductive apex shows a layering of cells similar to that in the cone dome. (D) Longitudinal section of one of the primordia of the antherophore, with periclinal divisions of the surface layer and a cell layer pattern similar to that in the reproductive apex. (E) Longitudinal section of the antherophore at the time of synangium initiation. (F) Cleared male 'flower' harvested in summer; each synangium is supplied by its own vascular bundle. Scale bars: A,E 200 µm; B–D 100 µm; F 4 mm. am = apical meristem; ap = antherophore; ca = cone apex; ct = central tissue; fb = fertile bract; lp = leaf primordium; mb = median bract; pt = peripheral tissue; ra = reproductive apex; sa = subapical group; sl = surface layer; sy = synangium; tb = transversal bract; vb = vascular bundle.

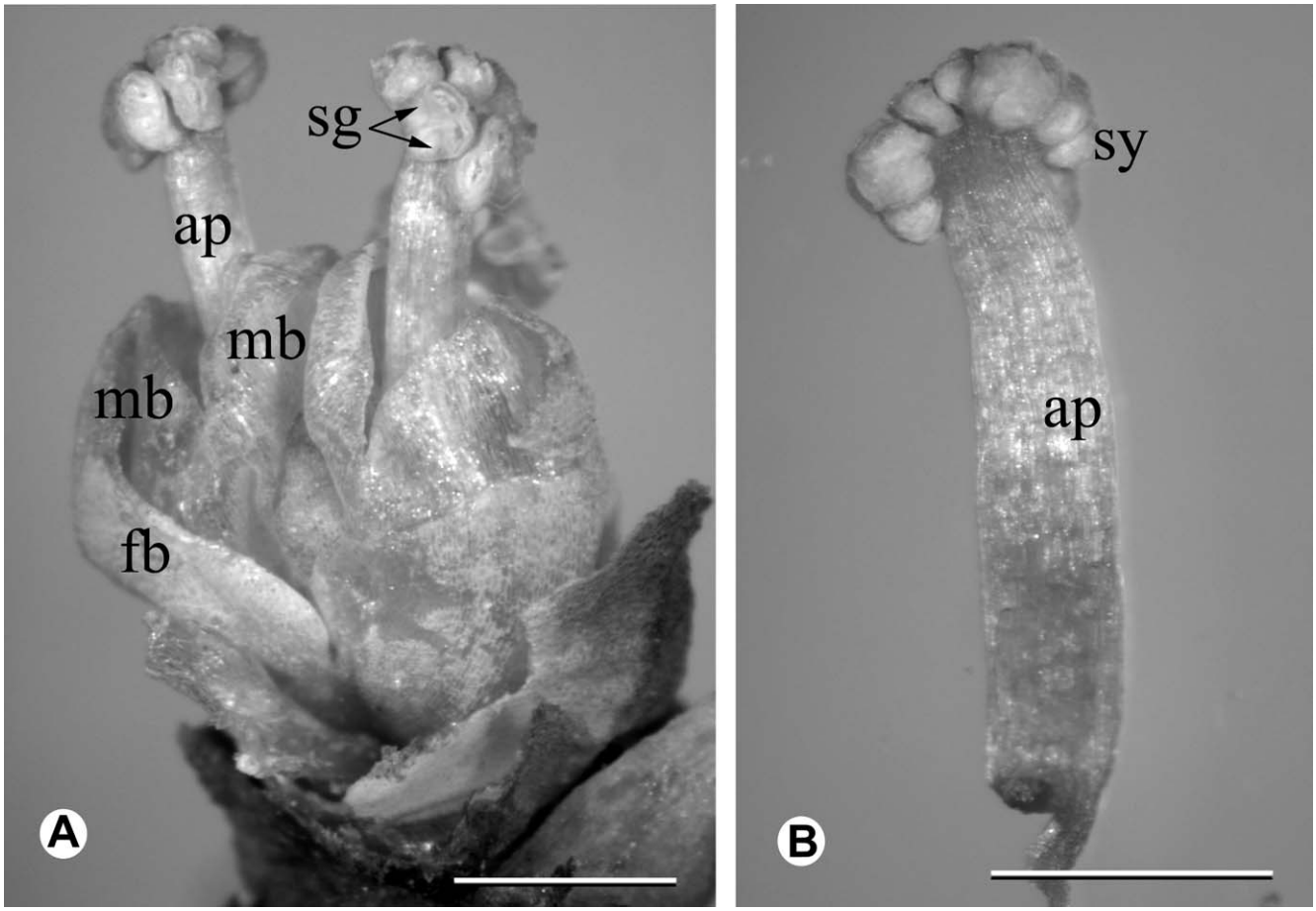


Fig. 5. *Ephedra distachya*: (A) Simple male cone with two antherophores that are surrounded by two median bracts. (B) Antherophore with about eight synangia, each consisting of two sporangia. Scale bars 1 mm. ap = antherophore; fb = fertile bract; mb = median bract; sg = sporangium; sy = synangium.

periclinal divisions of the dermal layer, which is best seen in longitudinal sections (Fig. 7B). The apex between the primordia stops growing and is used up by the developing primordia. Each primordium forms one half of the antherophore.

Immediately after the initiation of both halves, four primordia of the synangia are simultaneously initiated at the edge of each half (Fig. 6C and D). The apex of each half of the antherophore stops growing, and further growth of the antherophore is restricted to the developing synangia (Fig. 8A). This is also indicated by the less dense cytoplasm of the apical cells as opposed to the dense cytoplasm of the cells of the developing synangia (Fig. 7C).

At the end of winter, the primordia of the synangia begin to bifurcate, each forming two sporangia. In total, both halves of the antherophore form eight sporangia, which are arranged in two groups of four synangia each (Fig. 8B and C). The synangia are now about 200 µm long and 100 µm broad. Longitudinal sections show that the bifurcation of the synangium originates from the initiation of the two sporangia. The cells of the developing sporangia display dense cytoplasm (Fig. 7D), whereas the cells between both sporangia start to vacuolate so that the connecting line of the synangia is formed. The dermal layer of the sporangia shows only anticlinical cell divisions at that time. Thus, the sporogenous tissue of the sporangia originates from hypodermal layers (Fig. 7E).

Although initiation of the synangia of each half of the antherophore occurs simultaneously, the connection lines of the synangia display a decussate arrangement. The arrangement of the connection lines of the median synangia is perpendicular to that of the synangia in transversal position (Fig. 8C). During further development, the sporangia enlarge and the dehiscence slits are formed. The orientation of each dehiscence slit is perpendicular to the connecting line of the synangia (Fig. 8D). The stalk of the antherophore is elongated by an intercalary meristem at the bases of the synangia. At anthesis, the stalk is about 3 mm long, the sporangia about 500 µm. Each synangium is supplied by its own vascular bundle (Fig. 7F).

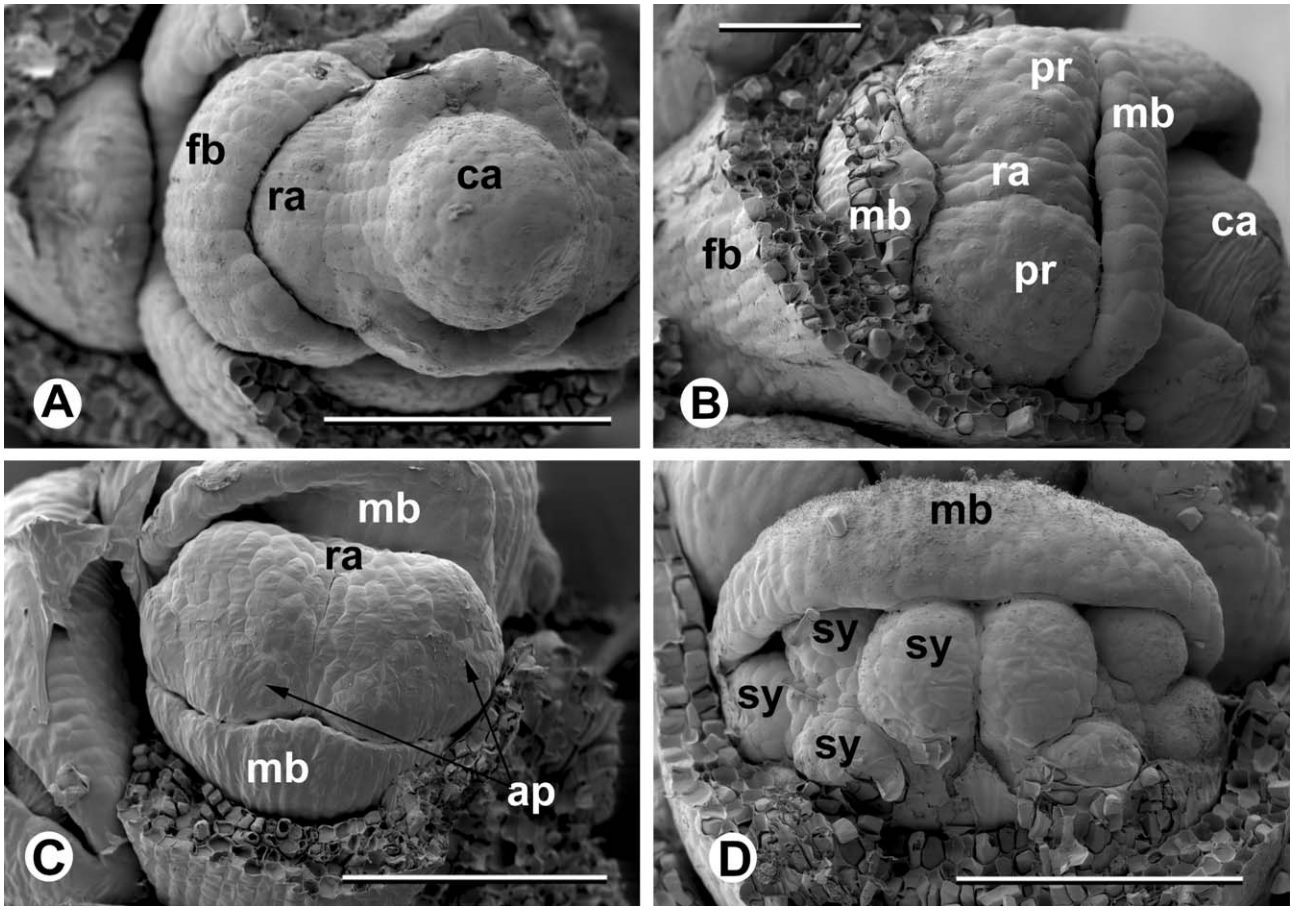


Fig. 6. *Ephedra distachya*, morphogenesis of male cones; SEM micrographs. (A) Cone apex in autumn, with several fertile bracts. (B) Reproductive apex at the time of initiation of median bracts, in transversal position the two parts of the antherophore are formed. (C) The reproductive apex stops growing and the two parts of the antherophore initiate the synangia. (D) Each half of the antherophore develops four synangia in a whorled arrangement. Scale bars: A,B 100 μm, C,D 200μm. ap = antherophore; ca = cone apex; fb = fertile bract; mb = median bract; pr = primordium of one half of the antherophore; ra = reproductive apex; sy = synangium.

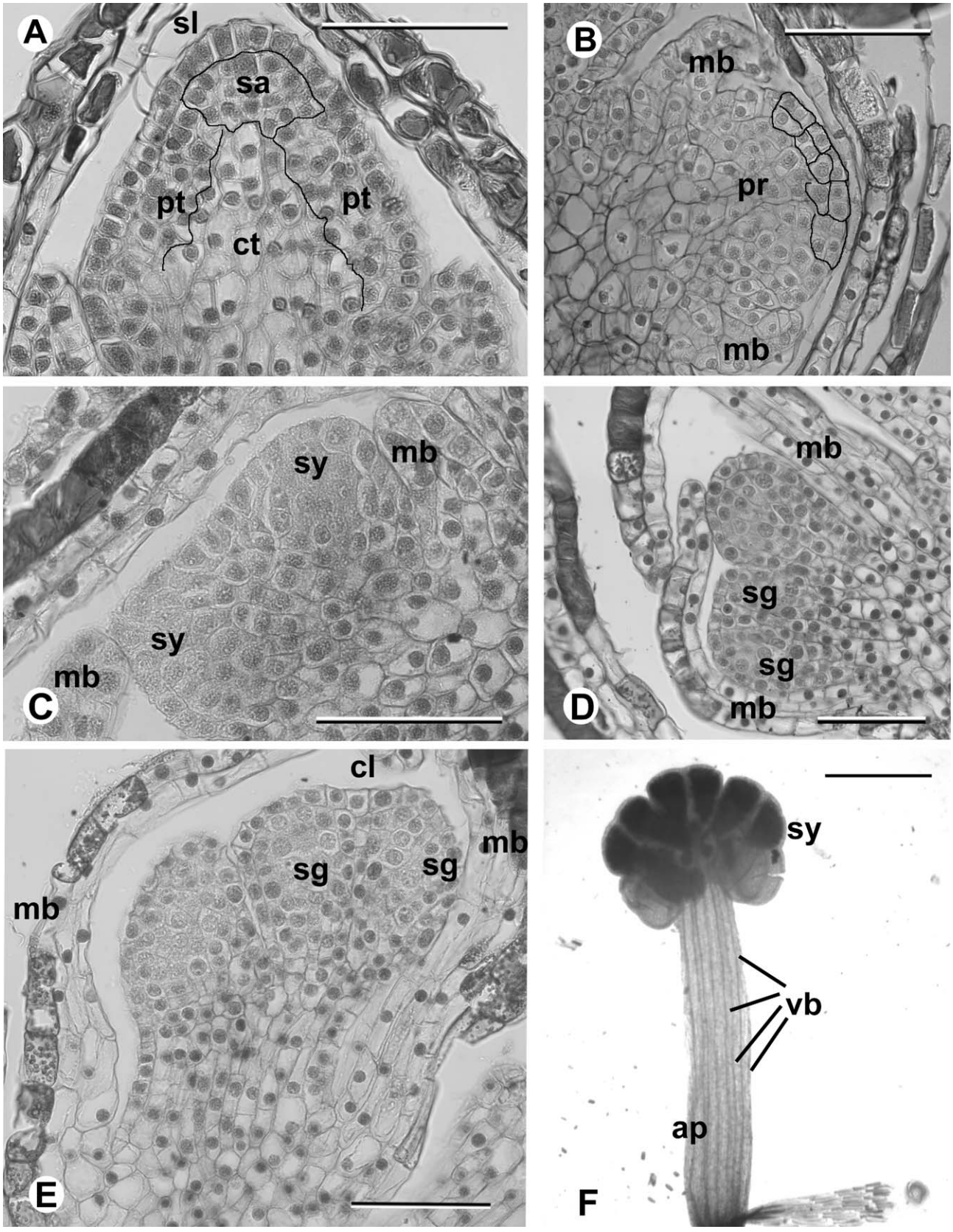
Discussion

Interpretation of the male antherophore of *Welwitschia mirabilis*

At present, it is mostly accepted that the male 'flower' of *W. mirabilis* consists of two pairs of sterile bracts, one pair of male sporangiophores, and a sterile ovule in terminal position (Martens 1971; Endress 1996; Hufford 1996). This interpretation is based on the observation by Martens that the morphogenesis of the antherophore of *W. mirabilis* begins from two distinct, separate primordia continuing the decussate phyllotaxis of the male 'flower'. Our results confirm this observation (Fig. 2D), thus an interpretation of the antherophore as one or two whorls of anthers, as assumed by Hooker (1863) and Church (1914), is not supported.

Although it seems quite plausible to regard one-half of the antherophore as a pinnate sporangiophore with stalked synangia in terminal position, morphogenesis of both halves is completely different from that of male sporangiophores in other gymnosperms. In conifers (Erspamer 1952; Mundry 2000) as well as in cycads (Smith 1907; Mundry and Stützel 2003), the primordia of the sporangiophores at first differentiate a prominent tip (more or less phylloid), and only afterwards the initiation of the sporangia or synangia begins. Even the non-phylloid sporangiophores of *Taxus* begin their development with the differentiation of the primordial tip of the sporangiophore (Mundry and Mundry 2001).

By contrast, the stalked synangia of *W. mirabilis* arise at the base of an undifferentiated conical tip (Fig. 4A). Although lacking a subtending bract, the conical shape and the central position of the initially remaining tip of both halves are strikingly similar to a reproductive apex. Moreover, the later abortion of this structure is typical for apices that have finished their development but unusual for sporangiophores or leaves. It is worth noting that the conical apex of the primordium was described by Martens (1971) as a pseudostamen,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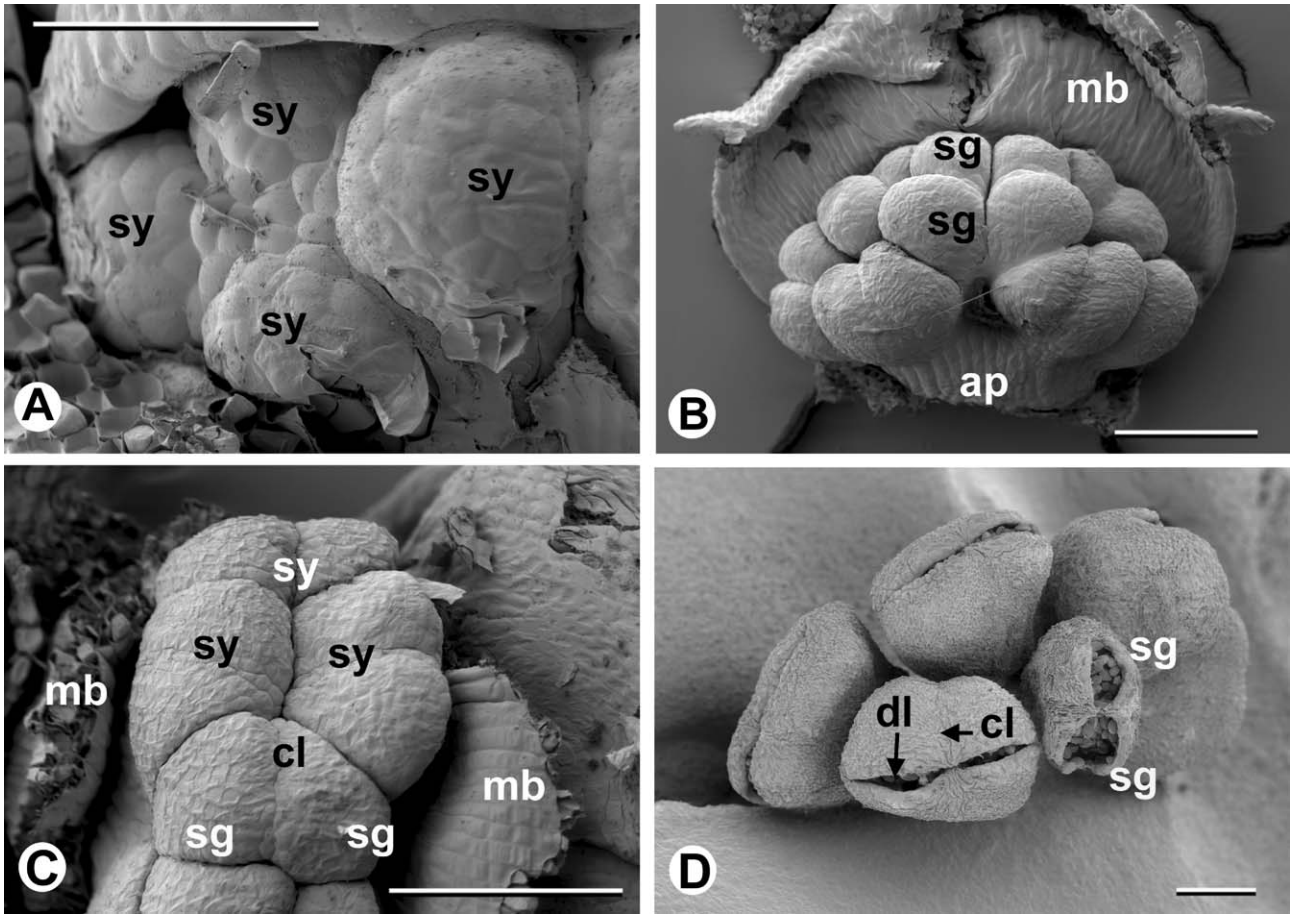

Fig. 8. *Ephedra distachya*, morphogenesis of the antherophore; SEM micrographs. (A) Detail of one half of antherophore at time of initiation of synangia; the four synangia arise in a whorl. (B) The young synangia bifurcate, each forming two sporangia. (C) The connecting lines of the sporangia reflect the decussate arrangement. (D) The dehiscence lines of the sporangia are perpendicular to the connecting line. Scale bars: A 100 µm, B–D 200 µm. ap = antherophore; cl = connecting line; dl = dehiscence line; mb = median bract; sg = sporangium; sy = synangium.

although usually neither fertile nor sterile synangia originated from this structure. A further kind of pseudostamen is described by Hufford (1996) from nearly mature material, but this seems to be a different structure, because the pseudostamens in the centre of the stalked synangia are usually aborted during development (Fig. 4C).

Although early developmental stages of leaf and shoot primordia of gymnosperms are very similar within a species (von Guttenberg 1961), the histological pattern of the primordia gives further support for the shoot character of both halves, for which the late differentiation of a peripheral and a central tissue zone seems to be a good indication (Fig. 3B–D). Neither the bracts nor the synangia show a layering into a peripheral and a central tissue zone. Furthermore, a histological pattern more similar to the leaves is shown by the primordia of the stalked synangia, as the dermal layer in the

Fig. 7. *Ephedra distachya*, morphogenesis of male cones; LM micrographs. (A) Longitudinal section of cone apex, showing differentiation into subapical group, peripheral and central tissue; surface layer displays only anticlinal divisions. (B) Longitudinal section of one primordium of the antherophore, showing that growth originates from several periclinal divisions of the surface layer. (C) Subsequent developmental stage of one part of the antherophore, showing initiation of synangia from hypodermal layers. (D) Longitudinal section of a later developmental stage, showing that sporangia also originate from hypodermal layers. (E) The antherophore elongates by an intercalary meristem at the bases of the synangia. (F) Cleared male antherophore harvested in spring; each synangium is supplied by its own vascular bundle. Scale bars: (A–C) 100 µm, (D,E) 200 µm, (F) 1 mm. ap = antherophore; cl = connecting line; ct = central tissue; mb = median bract; pr = primordium of one half of the antherophore; pt = peripheral tissue; sa = subapical group; sg = sporangium; sl = surface layer; sy = synangium; vb = vascular bundle.

developing synangia displays a few periclinal walls at an early stage, and later only anticlinal divisions, similar to the development of cone bracts.

Another typical feature of the simple sporangiophores is shown by the pattern of the vascular system. Each stalked synangium in *Welwitschia* is supported by its own single-stranded vascular bundle down to the base of the antherophore (Fig. 3F). Moreover, the results of Martens (1971) indicate that each vascular bundle enters the 'floral' axis separately: first the lateral and then both median bundles of each half. This would also be unusual for a pinnate structure, which usually has a branched vascular system before entering the axis it descends from.

Therefore, we propose that one half of the antherophore is homologous to a reduced, lateral male cone that has lost its subtending bract (Fig. 9A and B). Following this argumentation, the stalked synangia represent the sporangiophores, which are thus simple with a terminal synangium of at least three sporangia. Accordingly, the male 'flower' of *Welwitschia* would represent a structure analogous to angiosperm pseudanthia (e.g. as in Triuridaceae, see Rudall 2003).

As further indications for the compound character of the *Welwitschia* 'flower' two lateral mounds, observed by Martens (1961) in submature material at the base of the sterile ovule, have been used (Lignier and Tison 1911). Martens interpreted these structures as homologous to the wings of the female cones. In morphological studies, these mounds have not been detected. Lignier and Tison (1911) homologized these lateral structures with the median bract located below the antherophore, and still assumed that the male 'flower' is homologous to a compound strobilus. In contrast, Hufford (1996) notes that these mounds do not fit in the phyllotaxis, and regards this as an argument against the hypothesis of the 'flower' representing an 'inflorescence'. However, these mounds neither fit in the 'flower' nor in the 'inflorescence' concept, and thus cannot be used as support for either hypothesis.

A difficulty in the interpretation of our results is that it is unclear whether the envelope of the antherophore (the later median bracts) begins from primordia in median or transversal position (arrows, Fig. 2B). The same is true for the female 'flower' of *Welwitschia*. The wings of the ovules seem to start mostly from median primordia (Martens 1971; Mersmann 1998), but at a later stage a ring-like structure continues to grow predominantly from the transversal parts forming the prominent lateral wings of the seed. Several authors assumed that the position of the wings is lateral, but this would also disagree with the phyllotaxis, because the female 'flower' begins with two bracts in lateral position. One possible solution could be that the envelopes are formed by two successive whorls unified into a tube, and thus the lateral wings of the female 'flower' would fit in the phyllotaxis as the assumed lateral male cones (Fig. 9C and D). Accepting that the sterile ovule is a reduced female 'flower', the lateral mounds would be homologous to the wings consisting in this model of two pairs of bracts, and consequently they would also fit with the phyllotaxis.

This would explain the somewhat ambiguous results from morphological studies (Lignier and Tison 1911; Martens 1971; Hufford 1996), although there is no evidence from vegetative parts that such a union of the organs of two subsequent whorls could be expected. Nevertheless, the absence of a prominent subtending bract remains problematic in our interpretation, although the reduction of subtending bracts is quite common for the evolution of 'pseudanthia'.

Interpretation of the male antherophore of *Ephedra distachya*

Based on the observation by Eames (1952) that a residual apical projection is located between the halves of the antherophore, it is mostly accepted that the male antherophore of *Ephedra* consists of two sporangiophores instead of one terminal one. Hufford (1996) thus assumed that the male 'flowers' in *Welwitschia* and *Ephedra* show basically the same bauplan, although the male sporangiophores in *Ephedra* sometimes exhibit more synangia (stalked or sessile) and no terminal sterile ovule.

In contrast to these studies, Fagerlind (1971) emphasized the lack of any apical rudiment, based on his anatomical studies on the male cones of *Ephedra*. As a result he denied the leaf character of the halves.

Our results show clearly that an apical meristem is located between the halves (Fig. 6B), confirming the observations by Eames (1952). In some of the cones investigated the apex was not conspicuous, and we can assume its loss in derived species or in weakly developed 'flowers'. Nonetheless, our results indicate that the halves of the antherophore in *Ephedra* represent two separate structures, and consequently it is quite obvious to assume that the male 'flowers' of *Welwitschia* and *Ephedra* are homologous structures as assumed by Hufford (1996).

Following our interpretation, based on the morphogenesis of the antherophore of *Welwitschia*, each half of the antherophore of *E. distachya* also represents a lateral male cone with four whorled, simple sporangiophores (Fig. 10A and B). In *E. distachya* the synangia are sessile or very shortly stalked, but in certain other species they show a conspicuous stalk. Therefore, the whorled arrangement of the simple sporangiophores could easily be derived from the decussate phyllotaxis of *Ephedra*, as seen by the crosswise orientation of the synangia and dehiscence slits (Fig. 10B).

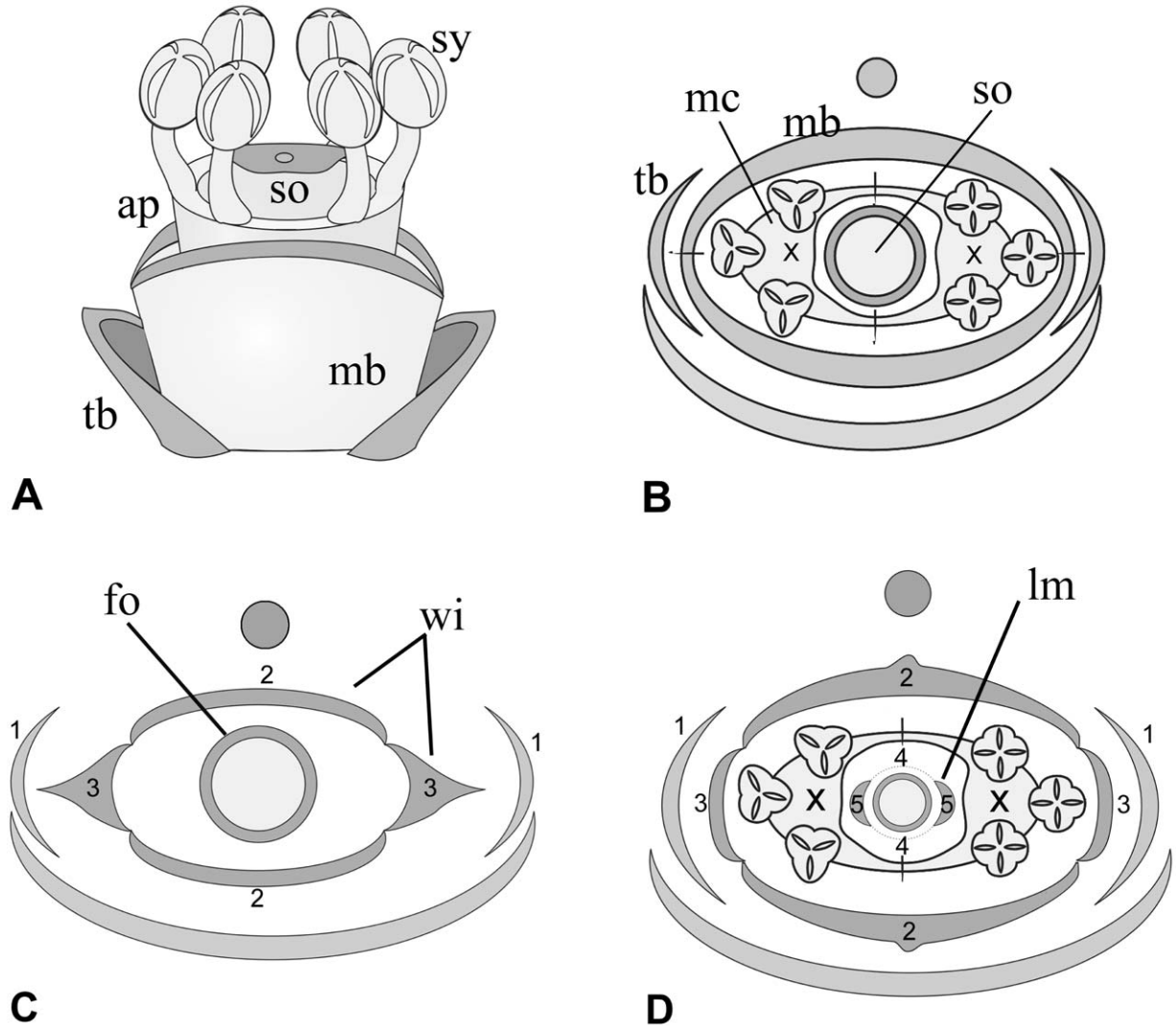

Fig. 9. *Welwitschia mirabilis*: (A) Upright projection of the male 'flower'. (B) New interpretation of the male 'flower'; both halves of the antherophore represent a lateral male cone at right angles to the median bracts. (C) Interpretation of the wing of the female 'flower' as composed of two pairs of bracts (2, 3). (D) Interpretation of the envelope (median bracts) of the male 'flower' as composed of two pairs of bracts (2, 3), both lateral bracts would be the reduced (3) subtending bracts of the lateral male cones; the sterile ovule in the centre represents a reduced female 'flower' as indicated by the two lateral mounds (5), which are homologous to the lateral bracts of the wing of the fertile ovule; the median bracts of the female flower are reduced as indicated by the dotted line (4). ap = antherophore; fo = fertile ovule; lm = lateral mound; mb = median bract; mc = male cone; so = sterile ovule; sy = synangium; tb = transversal bract; wi = wing of the fertile ovule.

In contrast to the results on *Welwitschia*, a conical apex in the centre of the four assumed sporangiophores is absent, and the whole apex of each half is completely used up by the developing synangia. Moreover, the synangia of *E. distachya* are sessile and not stalked like the synangia of *W. mirabilis*. Therefore, the homology of the sessile synangia and the simple sporangiophores is not obvious at first glance, but still seems possible considering the stalked synangia in certain other *Ephedra* species (Stapf 1889; Cutler 1939). Moreover, each sessile synangium of *E. distachya* exhibits a single vascular bundle down to the base of the antherophore (Fig. 7F) as do the stalked synangia of *Welwitschia*.

Although there is no further support for our interpretation from morphology, the histological pattern of the developing antherophore is of interest. A series of periclinal divisions like those of the primordia of the halves (Fig. 7B) is not observed in developing leaves, nor in vegetative apices (von Guttenberg 1961), but was described in studies of the female cones by Takaso (1984). In this study, the simultaneous periclinal

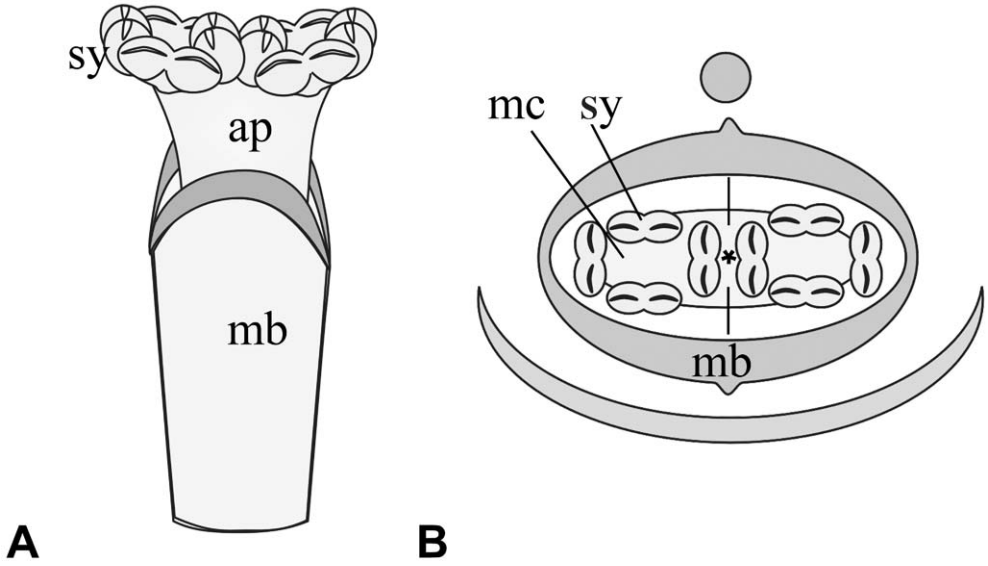

Fig. 10. *Ephedra distachya*: (A) Upright projection of the male antherophore surrounded by the median bracts. (B) New interpretation of the male 'flower', with each half representing a lateral male cone; likewise, the synangia are arranged in a decussate manner. ap = antherophore; mb = median bract; mc = male cone; sy = synangium; ∗ = abortive apex.

division of dermal cells is shown for the apices of female cones arising in the axils of the cone bracts.

Although this pattern was also described for male cones of *Ephedra* by Fagerlind (1971), the concordance in both sexes has not been regarded as significant, because both structures are usually interpreted as different kinds of organs. If our assumptions on *Welwitschia* are true, the striking similarity is not accidental and gives further support to our interpretation.

General conclusions for the Gnetales

Following our interpretation, the male 'flower' of Gnetales represents an 'inflorescence' (compound cone), with lateral male cones displaying simple sporangiophores each consisting of one terminal synangium. Regarding the frequent occurrence of hermaphrodite compound cones in some species of *Ephedra*, and the terminal sterile ovule in *Welwitschia*, we can assume that the ancestor of Gnetales displayed branching systems with distal female cones and basal male cones (Fig. 11A–C).

Beginning with such an ancestor, the first step is the evolution of the antherophores by condensation of the basal, lateral male cones combined with the loss of their subtending bracts. The loss of the stalks of the sporangiophores would explain the sessile appearance of the synangia in most *Ephedra* species (Fig. 11D and E). The partly remaining female cones in distal position would explain the occurrence of hermaphrodite compound cones in *Ephedra* and the terminal sterile ovule in *Welwitschia* (and the lateral mounds as discussed above, Fig. 11F and G). This interpretation implies different kinds of synorganization between the 'flowers' of *Welwitschia* and *Ephedra*, although the antherophores are homologous.

Although we have not yet investigated the reproductive structures of *Gnetum* in detail, the occurrence of the highly condensate and hermaphrodite cones of *Gnetum* also supports such a transformation series. Therefore, our interpretation of the reproductive structures could explain the differences in the morphology of Gnetales, although it remains necessary to verify our interpretation by re-examining the female reproductive structures and morphology of *Gnetum*.

Evolutionary implications of the new interpretation

The male sporangiophores of Gnetales are different from other gymnosperm sporangiophores. They share with the male sporangiophores of the cycads the occurrence of synangia and, following the classical interpretation (Martens 1971; Hufford 1996), a pinnate arrangement, but in spite of these similarities, the two groups differ in positions of the synangia and in morphogenesis of the sporangiophores (Mundry and Stützel 2003). Neither in the classical view nor in our interpretation are gnetalean synangia located on the abaxial side of lateral leaflets as in the cycads. Therefore, a close relationship between both types of sporangiophores is unlikely.

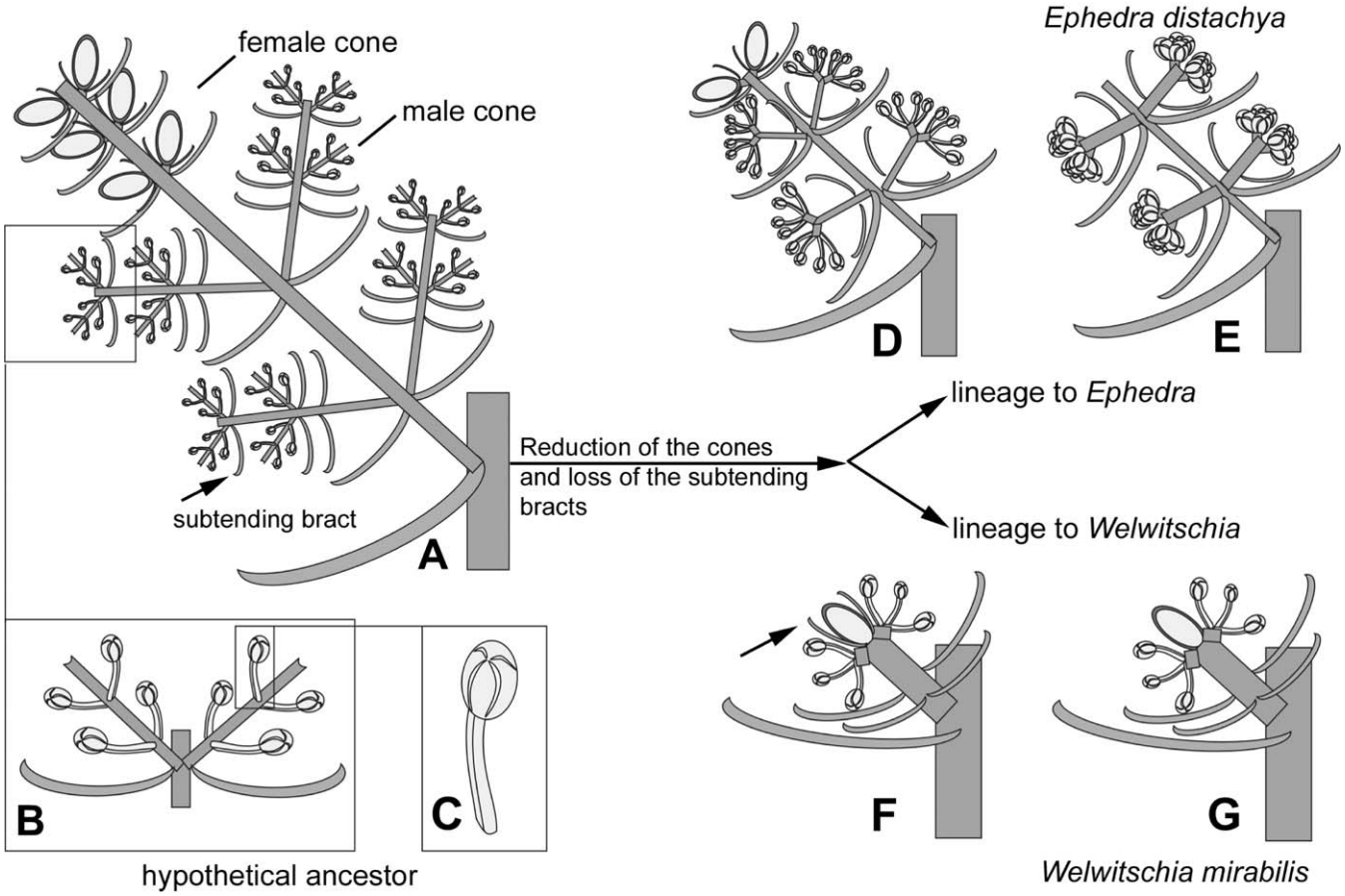

Fig. 11. Transformation series from a hypothetical ancestor of Gnetales to the male 'flowers' of *Welwitschia* and *Ephedra*. (A–C) Hypothetical ancestor of the Gnetales, displaying distal female and basal male cones lateral to the main axis (A), compound male cones with simple sporangiophores that are arranged decussately (B), bearing sporangiophores with at least four sporangia (C). (D,E) Lineage to the species of *Ephedra*: intermediate stage (D) with stalked synangia and hermaphroditic compound cones, terminal stage (E) with sessile synangia and monosporangiate, compound cones. (F,G) Lineage to *Welwitschia mirabilis*: intermediate stage (F) with the two lateral mounds (arrow) as a relict of the compound female cones; *W. mirabilis* (G) with true 'pseudanthium'.

The male sporangiophores of conifers (with the exception of some Taxaceae) are usually regarded as simple with a more or less distinct phylloid tip that bears the sporangia on the abaxial side (Erspamer 1952; Mundry 2000). The assumed pinnate sporangiophores of Gnetales with synangia on the adaxial side show no similarity to those of conifers. Following our interpretation, both groups display simple sporangiophores, although the lack of any kind of phylloid tip conflicts with a close relationship between conifers and Gnetales as proposed on the basis of molecular studies. If Gnetales are nested within conifers, this would imply that the phylloid tip of the conifer sporangiophores has independently evolved twice within conifers. Otherwise, it is possible that Gnetales have lost a phylloid tip, although no structural evidence for such a hypothesis has been reported.

The superficial similarity between angiosperm stamens and the sporangiophores in *Welwitschia* cannot nevertheless be used as an argument for an anthophyte hypothesis. Accepting that the 'flower' of *Welwitschia* is derived from a 'pseudanthial' structure, its structure is even farther apart from angiosperm flowers than previously thought. Thus, our morphological results coincide with recent molecular studies rejecting a close relationship between angiosperms and Gnetales (e.g. Bowe et al. 2000; Chaw et al. 2000; Frohlich and Parker 2000; Schmidt and Schneider-Poetsch 2002; Winter et al. 1999).

The morphological interpretation of putative gnetalean fossil sporangiophores also conflicts with our interpretation of extant structures. The arrangement of several hundred trisporangiate synangia on the assumed adaxial side in *P. kuespertii* is completely different from a simple sporangiophore with one terminal synangium. Admittedly, the adaxial position is just assumed (van Konijnenburg-van Cittert 1992) and the macromorphology shows a striking similarity to cycad sporangio-

phores, in which the synangia are located at the abaxial side. Furthermore, the occurrence of ephedroid pollen does not stringently prove a gnetalean affinity. Other putative gnetalean male cones like *M. clathratus* also differ from a simple sporangiophore with a terminal synangium, although not as dramatically as *P. kuespertii*. However, the sporangia of *M. clathratus* are not arranged in synangia, and instead of being located in terminal position they are on the abaxial side of a sterile hump.

Apart from several further fossils with uncertain affinity to Gnetales, we want to focus on another fossil group, Cordaitales, in which the synangia are placed on simple sporangiophores, although the terminal synangia are fused only basally. Eames (1952) proposed the homology of the male sporangiophores of Gnetales and Cordaitales. However, he interpreted the male sporangiophores in the classical fashion, as pinnate. Cordaitales are usually regarded as sister to conifers, based mainly on the work by Florin (1950). Although the exact relationship between both groups is controversially discussed (Rothwell 1988), the affinity between conifers and Cordaitales could be a question crucial to the reconstruction of gnetalean phylogeny.

The pollen cones of *Cordaitanthus concinnus* are compound with four-ranked subtending bracts (Rothwell 1988). In the axils of the bracts, secondary axes arise that display helically arranged scales, the distal ones with terminal sporangia. Following our interpretations on Gnetales, the male reproductive structures of both groups would share several features, such as simple sporangiophores, compound cones, and terminal position of the sporangia, which are partly fused in Cordaitales and completely fused in Gnetales. This would support a close affinity between Gnetales and Cordaitales, and consequently the results of recent molecular studies would become congruent with morphological aspects, if conifers and Cordaitales are truly closely related.

However, it is not easy to link the male reproductive structures of extant conifers to those of Gnetales and Cordaitales. The pollen cones of all extant conifers are usually regarded as simple, displaying simple sporangiophores with abaxial attached sporangia, although the occurrence of compound male cones in the Taxaceae (including Cephalotaxaceae) is regarded as primitive for conifers by some authors (Wilde 1975; Mundry and Mundry 2001).

Following the interpretations by Florin (1950), male cones of fossil conifers would be similar to extant ones, with simple cones bearing sporangiophores with sporangia attached on the abaxial side. In contrast, in recent years several fossil male conifer cones have been described with sporangiophores that differ markedly from the extant ones. Several Paleozoic and Triassic male cones exhibit sporangia connected to the adaxial side of the sporangiophore (Grauvogel-Stamm and Galtier 1998; Mapes and Rothwell 1998; Kerp et al. 2001; Rothwell and Mapes 2001). Grauvogel-Stamm and Galtier (1998) demonstrate that the Triassic male cone *Darneya* is compound with sporangiophores that represent subtending bracts bearing clusters of stalked sporangia. In contrast to the uniformity of the extant conifer sporangiophores, these examples illustrate the great diversity among fossil conifers (Grauvogel-Stamm and Galtier 1998; Mapes and Rothwell 1998).

Of relevance in this context is the Paleozoic male cone of *Thucydia mahoningensis*, described by Hernandez-Castillo et al. (2001). This male cone exhibits a compound arrangement with lateral, simple dwarf shoots that consist of sterile scales and single sporangia (simple sporangiophores). Hernandez-Castillo et al. (2001) emphasized that this morphology supports the close relationship of conifers, Cordaitales and Gnetales.

Accepting our interpretation of reproductive structures of Gnetales, the common ancestor of all three groups displayed compound male cones composed of simple sporangiophores with a terminal cluster of free sporangia. In Cordaitales these sporangia are fused basally, in Gnetales they are fused completely to form one terminal synangium. Such an ancestor could also be the starting point for the different lineages of conifers. The fusion of the terminal clusters with the subtending bracts is reminiscent of the male cone *Darneya*, as described by Grauvogel-Stamm and Galtier (1998), and the reduction in sporangia number is illustrated by the single sporangium of *Thucydia mahoningensis*. The evolution of the fossil sporangiophores with sporangia on the adaxial side remains unexplained in this interpretation; unless they were transformed in the same way as described for *Darneya*. Also unexplained is the occurrence of the phylloid tip of extant conifer sporangiophores, and further investigations on extant and fossil conifers are required for a convincing concept.

Nonetheless, our results and interpretation of gnetalean sporangiophores open a new approach to the phylogeny of this group, combining the results of molecular, morphological, and palaeontological data. Many problems remain, e.g. an explanation for the divergent fossil record of conifer pollen cones, or the evolution of the phylloid tip of sporangiophores with abaxial sporangia. Moreover, it appears necessary to reconsider female reproductive structures and to investigate the early morphogenesis of further species of *Ephedra* and *Gnetum* in order to develop a more convincing concept on the evolution of gymnosperms. Nevertheless, the interpretation presented here for gnetalean sporangiophores as being simple with one terminal synangium may resolve many conflicts between molecular and morphological studies that have arisen in the last two decades.

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